

a motor effect on the pregnant human uterus, and that, therefore, the use of sympathomimetic substances in labour is justified.

Summary.

The movements have been recorded of the isolated non-pregnant human uterus and Fallopian tube. Adrenine has a powerful motor action on both these organs. The deduction is drawn that this is the qualitative effect of sympathetic innervation of the human uterus, at least when non-pregnant.

Pituitrin also stimulates the human uterus proper to contraction, but no such effect has been definitely obtained on the Fallopian tube.

The Presence of Inorganic Iron Compounds in the Chloroplasts of the Green Cells of Plants, considered in Relationship to Natural Photo-synthesis and the Origin of Life.

By BENJAMIN MOORE, D.Sc., F.R.S., Professor of Biochemistry, University of Liverpool.

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(From the Johnston Biochemical Laboratory, University of Liverpool.)

It has been demonstrated by Moore and Webster* that colloidal solutions, or suspensions, of salts or oxides of iron, in presence of dissolved carbon dioxide and with the energy supply of sunlight, possess the power of synthesising formaldehyde. Since this is known to be the first step in the process of organic synthesis of the substance of all living plants and animals from inorganic material and must, moreover, have occurred in past ages over immense areas of the earth's surface before life began to exist on the planet, the conclusion was drawn that life must have originated by continual development of more and more complex organic substances from this simple commencement.

It is in this first stage of all that the greatest production of chemical energy occurs, and accordingly a transformer of light energy into chemical energy is essential. Although the more highly organised carbohydrates and proteins still require catalysts for their synthesis, weight for weight they contain scarcely any greater storage of chemical energy than formaldehyde,

* 'Roy. Soc. Proc.,' B, vol. 87, p. 163 (1913).

and once an accumulation of organic material has been reached, even the fats and their allies can easily be synthesised by the combination of linked exothermic and endothermic reactions by the living cell acting as a transformer, without the use of external energy such as that of sunlight.*

Once organic matter has been synthesised, the living cell can oxidise one portion of this to reduce still more another portion. In this manner the animal cell can oxidise carbohydrate, for example, and use the energy so set free to build up another portion of carbohydrate into fat which weight for weight contains double as much chemical energy as the carbohydrate, without in the process making use of an external source of energy.

At the commencement, however, when only water and carbon dioxide are the available materials, it is indispensable that an external source of energy such as sunlight should be available, and a suitable mechanism, or chemical system, for the transformation of this store of energy into the chemical energy of organic compounds.

Such a transformer has been recognised for a long period in the chloroplast or chlorophyll-granule of the green cell of the higher plant.

Since the days of de Saussure,† now over a century ago, the green colouring matter of the leaf, chlorophyll, has been regarded as the fundamental agent for this world-wide photo-synthesis. But it is remarkable how completely this view is based upon indirect or circumstantial evidence, and how little, if any, direct observation can be cited in its support.

Chlorophyll is known by the biochemist to be one of the most complex of substances, comparable to hæmoglobin in its molecular structure, and yielding a host of disintegration constituents themselves complicated substances of high molecular weight. Between the simple colloidal molecules of inorganic iron salts in solution or suspension and such a highly complex organic substance as chlorophyll there is a wide hiatus, and it was with the view of discovering some intermediate links or finding some explanation for the gap that the present experiments were commenced.

Before describing the experiments in detail it is desirable to touch upon present views as to photo-synthesis in the green cell as far as these bear upon our investigation, in order to give an appropriate setting to the new facts, and show how present knowledge regarding the absolute necessity for the presence of iron in the green leaf, which has been hitherto devoid of all explanation, led up to these experiments.

Photo-synthesis with production of oxygen only occurs in the chlorophyll-

* See Moore, 'Recent Advances in Physiology and Biochemistry,' edited by Leonard Hill, pp. 135, 138, Arnold, London (1906).

† "Recherches Chimiques sur la Végétation" (1804), 'Ostwald's Klassiker,' Nos. 15-16

containing parts of the plant, and only in these when they are exposed to light. Also, when a plant is allowed to grow in darkness, the leaves are found to be pale-yellow in colour, or chlorotic, instead of green. When a plant grown in darkness, and, as a result, possessing chlorotic leaves, is then exposed to light, the pale-yellow colour is rapidly replaced by a green, and then photo-synthesis is readily demonstrable by the evolution of oxygen and the appearance of starch granules.

The above reasoning constitutes the whole of the evidence that chlorophyll is the primary cause in the first act of photo-synthesis. It is to be observed that the entire chain of evidence is inferential, and that in order to form a valid proof, chlorophyll would require to be the only substance present in the chloroplast, which is very far from being the case. No observer has ever obtained an appreciable and satisfactory synthesis with pure chlorophyll in solution or suspension when removed from the other constituents of the chloroplast. Certain observers have observed minute traces of formaldehyde formation with chlorophyll solutions or emulsions, but even these traces of photo-synthesis have been stoutly denied by other competent observers. In any case, the photo-synthetic effect produced is infinitesimally small compared to that observed in the intact green cell.

The most recent and careful experiments upon this subject are those performed by Usher and Priestley* and by Schryver.† Usher and Priestley found that when a chlorophyll-containing extract from green leaves was spread out as a film or emulsion on a gelatine plate, small, but distinctly demonstrable amounts of formaldehyde were formed on exposure to sunlight. But in this case there is gelatine and the inorganic colloids it contains shown by its ash to be present, and in the chlorophyll extract there would undoubtedly be iron salts present, because about one-fourth of the iron of green leaves comes away in the alcoholic extract.

Schryver worked with an ethereal solution of chlorophyll allowed to evaporate at room temperature on a strip of glass, and found that although such films of chlorophyll on glass produced no formaldehyde in darkness even in presence of moist carbon dioxide, a minute amount of formaldehyde was formed when the film was exposed to sunlight even in absence of carbon dioxide, and a distinct reaction when the film was exposed to sunlight in presence of moist carbon dioxide. The amount of formaldehyde formed in all such experiments is, however, very minute compared to the products of photo-synthesis under natural conditions by the complete chloroplast.

* 'Roy. Soc. Proc.' B, vol. 77, p. 369 (1906); B, vol. 78, p. 318 (1906); B, vol. 84, p. 101, (1911).

† 'Roy. Soc. Proc.' B, vol. 82, p. 226 (1910).

Now the chloroplast contains a great deal more than chlorophyll, and when all the chlorophyll has been removed by some such reagent as hot alcohol there remains behind a colourless body, the so-called stroma. The chloroplast after the extraction is still a solid looking body, and to all appearances the only thing that has happened is that a thin layer of green colouring matter has been removed. There is no shrinking or shrivelling up of the chloroplast.

There is accordingly no experimental evidence that the primary agent in the photo-synthesis may not be contained in the colourless part of the chloroplast, and the chlorophyll may be evolved at a later stage in synthetic operations induced by some constituent of the colourless part. The function of the chlorophyll may be a protective one to the chloroplast when exposed to light, it may be a light screen as has been suggested by Pringsheim, or it may be concerned in condensations and polymerisations subsequent to the first act of synthesis with production of formaldehyde.

All these views and others are possible, and the function of chlorophyll in the chloroplast remains for solution, but it has not been proved that chlorophyll is the primary causative agent in the photo-synthetic process where the chief energy uptake occurs with formation of formaldehyde.

There are other pieces of experimental evidence apart from the repeated failures to obtain satisfactory synthesis with isolated chlorophyll which go to indicate that chlorophyll is not the transformer in the first link of the synthetic chain.

In the first place chlorophyll itself is a product of photo-synthesis, and therefore there must be some active photo-synthetic substance present in the chloroplast before the chlorophyll appears which indeed first produces the chlorophyll by its activity.

When a yellow etiolated leaf taken from the darkness is exposed to the light it contains no chlorophyll, but photo-synthesis, in the absence of chlorophyll, sets in, and chlorophyll itself is one of the products, not the originator or agent, of this photo-synthesis. The period from first exposure to light to the appearance of chlorophyll is too short to determine whether oxygen production and starch formation commence before chlorophyll is formed.

In the next place Engelmann,* by the application of his ingenious method of the oxyphile bacteria has clearly demonstrated two important facts; first, that the chloroplast alone, even when displaced from the rest of the cell, can, in presence of light, go on synthesising and producing oxygen;

* 'Botanische Zeitung,' 1881, p. 446, and 1887, pp. 394, 410, 418, 426, 442, 458.

and, in the second place, a still more important point in our chain of evidence, namely, that certain leaves such as those of the yellow variety of elder, which do not produce chlorophyll when exposed to light but contain yellow chromatoplasts, cause synthesis and produce oxygen. These observations as to synthesis by healthy yellow leaves have been confirmed by other observers such as Tammes, Josopait, and Kohl.*

The strongest piece of evidence, however, that iron salts are more fundamental to photo-synthesis and take an earlier share in it than chlorophyll, is that furnished by that process frequently occurring in green leaves known as "chlorosis."

Chlorosis is a pathological condition of green leaves of considerable practical importance in arboriculture, and the discovery of its cause is, as Molisch states, one of the most interesting and beautiful in the history of plant physiology.

It was shown in 1843 by Eusebe Gris† that chlorosis naturally occurring in the leaves of shrubs or trees could be entirely removed either by applying dilute solutions of iron salts to the roots, or by placing the detached chlorotic branch in a dilute solution of iron, or even by painting the chlorotic leaf with a very dilute solution of an iron salt. In some cases within 24 hours, and in nearly all cases in a period of a week to 10 days, the green colouring matter developed in the leaves where none had been before.

These results have been often confirmed and have been extended by Salm Horstmar, A. Gris, and Sachs.‡ Molisch§ has, moreover, shown in a long series of experiments with different species of plants that all green plants, *even when fully exposed to light*, become afflicted with chlorosis and fail to develop chlorophyll when they are grown in a culture fluid especially made devoid of iron. As soon as the reserve store of iron always contained in the seed embryo and cotyledons has been exhausted in the primordial leaves, only chlorotic pale-yellow leaves are formed. These pale-yellow leaves rapidly turn green if minute quantities of an iron salt are added to the culture fluid, or even if the surface of the leaf be painted over with a dilute solution of an iron salt, as had been previously shown by Gris to be the case with

* Quoted by Czapek, 'Biochemie der Pflanzen,' vol. 1, p. 447.

† 'De l'Action des Composés Ferrugineux sur la Végétation,' 1843. See also 'Comptes Rendus,' vol. 19, p. 1118 (1844); vol. 21, p. 1386 (1845); vol. 23, p. 53 (1846); and vol. 25, p. 276 (1847).

‡ Salm Horstmar, 'Versuche über die Ernährung der Pflanzen,' 1856; A. Gris, 'Annales d. Scien. Nat.,' Series IV, vol. 7, p. 201 (1857); Sachs, 'Flora,' 1862.

§ Molisch, 'Die Pflanzen in ihren Beziehungen zum Eisen,' Jena, G. Fischer (1892). Many of the references given are quoted from this source.

pathologically chlorotic leaves. So that iron is as indispensable to the green leaf as it is to the red blood-corpuscle.

The remarkable thing, in view of this failure to develop chlorophyll in absence of iron, is that chlorophyll itself is shown by all the more recent researchers to be quite free from iron.* Chlorosis and its cure by iron salts has accordingly remained a puzzle to plant physiologists ever since the time of the discovery of Gris.† The experiments to be recorded below furnish, for the first time, a rational explanation of chlorosis and its cure. The iron salts are necessary for the formation of the colourless portion of the chloroplast, for when all the chlorophyll and other fatty bodies and pigments are removed from the chloroplast by extraction with alcohol, and the colourless chloroplastic residue is treated with the micro-chemical tests for inorganic iron, a positive reaction in unmistakable degree is usually given by the colourless residue of the chloroplast.

This inorganic iron in presence of sunlight must give rise to photosynthesis and production of formaldehyde which is then carried on into sugar and starches by other constituents of the chloroplast, and it is probably here, somewhere in the later processes, that the chlorophyll finds its function. The chlorophyll itself, as shown by the facts of chlorosis, its removal by administration of iron, and the presence of iron salts in the colourless part of the chloroplast, is a product of synthesis from colourless substances or from the light-yellow pigment. For the production of the chlorophyll under normal conditions, both the presence of iron and the energy of sunlight are essential.

The reason for the earlier erroneous view that the chlorophyll molecule contained iron was that a certain fraction of the iron compounds contained in the green leaf becomes extracted by the alcohol used in the first extraction of the leaf,‡ so that all crude chlorophyll extracts contain iron. This, however, disappears on treating the alcoholic extract with benzol, and the product of purer chlorophyll separating from the benzol fraction is iron-free. At the same time its spectrum and other physical properties prove it to be unaltered chlorophyll.

Other facts which show the importance of iron compounds in the green leaf are that leaves which are not deciduous annually, such as pine needles, contain more iron in their later years, and also in leaves of annual growth

* See Molisch, *loc. cit.*, and R. Willstätter u. A. Stoll, "Untersuchungen über Chlorophyll," Berlin, J. Springer (1913).

† See Czapek, 'Biochemie der Pflanzen.'

‡ According to early observations of Boussingault (*Agronomie*, vol. 5, p. 128) from one-quarter to one-third of the iron is removed by the alcohol.

the older the leaf is the more iron does it contain in its ash. Thus Boussingault found in the ash of young leaves of *Brassica* 2 per cent. of Fe_2O_3 , while old leaves contained in their ash 9.64 per cent. *Lactuca sativa* had in the young leaves 2.67, and in the old leaves 6.43 per cent. of Fe_2O_3 in the ash. Another point is the curious conservation, resembling that seen in the animal economy, of the iron of the leaf in the case of deciduous leaves. Before the leaf drops a good deal of the iron is re-absorbed and stored for future use. This is shown by analyses of the iron of the leaves of *Fagus sylvatica* made by Rissmüller* in successive months. The figures quoted give quantities of Fe_2O_3 in 100 parts of dried leaves collected at the times of year stated—

	May.	June.	July.	Aug.	Sept.	Oct.	Nov.
Oxide of iron.....	0.35	0.51	0.58	0.75	1.03	0.60	0.59

The gradual increase of iron content to a maximum followed by a fall as the leaves grow sere is very interesting.

It has been shown by Molisch (*loc. cit.*) that iron is an essential constituent for the growth of all plants, whether green or otherwise, but the saprophytic and parasitic plants which contain no chlorophyll require much less iron and, as a rule, contain much less in their ash. Our own experiments show that the histo-chemical reactions for iron develop much more slowly in the fungi and are much less intense in degree. These feebler reactions probably arise from organic compounds of iron slowly being decomposed in traces and setting free ionic iron. These organic iron compounds of the fungi are concerned with some other function than photo-synthesis or chlorophyll formation; they probably take a part in nuclear structures, for many nucleins are iron-containing, and, as has been shown by Macallum, after treating with acid alcohol to unmask the iron previously present in an organic form, the chromatin of nuclei always contains iron.

The reactions for inorganic iron are shown most markedly with the more lowly organised plants such as unicellular green plants occurring alone or in lichens, or in delicate algal threads, but when proper precautions are taken they can also be clearly demonstrated in the chloroplasts of the higher plants. The reactions are particularly well shown by the chloroplasts of aquatic plants, where, as is well known, the percentage of iron in the ash is also high.

These facts are in keeping with the natural order of evolution and are also in accord with other observations. For example, many algæ (such as

* "Ueber die Stoffwanderung in der Pflanzen," 'Landw. Versuchsstationen,' 1874 ('Just's Jahresbericht,' vol. 2, p. 849, 1874).

certain Confervæ and Cladophora) deposit around them a layer of yellow to rust-red colour consisting of mixed ferrous and ferric oxides; this is often actively secreted from waters containing only traces of iron.

A considerable number of lichens also secrete incrustations of the mixed oxides of iron to such an extent as to change their appearance to an iron-oxide or ochre colour, so that they have been termed by systematic botanists "formæ oxydatæ, ochraceæ" or "iron lichens." The iron-oxide forms a fine incrustation usually on the mycelium of the fungus. No association of this iron-oxide with a photo-synthetic function has ever been suggested, but in view of our present knowledge of the photo-synthetic activity of iron salts some investigation in this direction is highly desirable. It is an interesting observation of Molisch, from our point of view, that these "iron lichens" flourish exclusively on the oldest primitive rock-formations ("Urgestein"). They are never found upon chalk formations, but grow upon granite, gneiss, syenite, and porphyry. Molisch was unable to find inorganic iron in the other lichens, but this doubtless arose from the less delicate methods he had at his command at that time, and from the fact that the fatty bodies contained in the green cells of the alga of the lichen had not been removed. When the lichen is extracted with alcohol and Macallum's hæmatoxylin test then applied, the algal cells rapidly stain a deep blue-black, showing the presence of inorganic iron, while the hyphæ of the fungus only take on a brownish tinge during the same time, and only give a faint positive reaction at the end of some days or weeks.

It is somewhat remarkable that the presence of iron in the chloroplast should for so long have escaped discovery. The explanation probably lies in the fact that little attention has been given to the application to the green cell of the histo-chemical tests for iron since the discovery by Macallum of the more delicate hæmatoxylin iron test, as also to the delicacy of the chloroplasts to the more drastic earlier method used by Molisch, and to these factors may be added the difficulty with which some of the chemical reagents for iron penetrate the green cell, and the presence in the chloroplast itself of fatty and lipoidal substances which prevent the ingress of the water-soluble stains.

Macallum* in 1894 before his discovery of unmordanted hæmatoxylin as a reagent for iron, and using then ammonium sulphide in glycerine as a reagent, states that bacteria gave no evidence of an organic iron compound, but in the Cyanophyceæ the chromophilous portions of the "central substance" contain iron, and iron may be also demonstrated in the peripheral granules containing the so-called cyanophycin. At this period, Macallum

* 'Roy. Soc. Proc.,' vol. 57, p. 261 (1894).

was specially concerned in proving the presence of organic iron in the chromatin of the nucleus and was not searching for iron in the chloroplasts, so that the reference above to the presence of iron in the cyanoplasts of the Cyanophyceæ is highly interesting to-day.

Molisch (*loc. cit.*) used long immersion in *saturated* potassium hydrate as a preliminary method for setting free masked iron (*i.e.* organic iron) in available form for after-detection by potassium ferrocyanide and hydrochloric acid, and in the later testing used very strong hydrochloric acid (10 to 20 per cent.). Such drastic procedures are very dangerous, because the alkali breaks down the delicate chloroplasts, and may also itself contain iron salts in traces; also in the second place, as pointed out by Quincke,* such strong acid will fairly rapidly set iron free in inorganic or ionic form from the potassium ferrocyanide reagent, and this ionic iron reacting with the remainder of the reagent will give the Prussian blue colour. Molisch found more iron in the epidermis and fibro-vascular bundles of green leaves than in the green mesophyll, but as he himself admits "the potassium hydrate so disorganises the nucleus and chlorophyll-granules that one can conclude nothing as to the distribution of iron in the cell."

So far as we have been able to discover there exist no records later than the above in the literature of the subject on the occurrence of iron in the chloroplasts of the green cell, nor any information as to the form in which iron compounds are present. No investigations with the iron hæmatoxylin test of Macallum appear to have been made hitherto upon plants.

Experimental Methods.

In carrying out tests for the detection of inorganic iron in the chloroplasts, and in plant tissues generally, two points must be carefully borne in mind, first, the previous preparation of the tissue and its subdivision so that the parts possibly containing iron may be penetrated by the reagents used for the detection, and secondly, that the reagents be applied carefully so that false results are not obtained. Here care must be taken with the concentration of the reagents and the relative periods of time within which positive results are obtained.

In regard to the preparation of the tissues, if sections are to be cut, care must be taken that this is done with a clean burnished knife. Control experiments show that a clean steel knife leaves no iron on the section. But, in most cases, since the question at issue is not the structural arrangement but rather whether this or that constituent contains iron, it is better to work with finely teased or broken up tissues. For this purpose glass rods drawn

* 'Arch. f. Exp. Path. u. Pharm.,' vol. 37, p. 183 (1896).

out to a point were always used instead of steel needles, and also, in order to break up some of the green cells and set free the chloroplasts, a portion of the tissue in each case was still more broken up by turning upon it the blunt end of the glass rod and grinding it between this and the microscope slide on which it was being mounted.

In choosing tissues for examination, preference so far as possible is given to those where the chloroplasts are more conspicuous in size, and also in some cases, such as *Spirogyra*, delicate filaments were chosen which could, after extraction as described below with alcohol, be mounted without breaking up.

In certain cases, such as *Pleurococcus*, staining can readily be obtained without previous chemical preparation of the tissue, but, in the majority of cases, the lipoids present along with the chlorophyll in the chloroplast prevent the penetration of the stain, also the green colour modifies and masks the blue of the hæmatoxylin in Macallum's test. For this reason it is well to remove the lipoids and chlorophyll, and in many cases this is by no means an easy task. In some cases standing in cold alcohol removes the chlorophyll quite effectually and leaves the tissue colourless and ready for staining; but in other cases the tissue may be left at ordinary temperatures for days in alcohol, and this may even be followed by several extractions with ether, and still some of the green colour remains. After a good deal of experimentation the best extractive in these latter cases was found to be boiling alcohol.

The tissue, either partially teased with the glass points if it is bulky like the leaf of a higher plant or a piece of lichen or moss, or left intact if a delicate structure like an algal filament or *Pleurococcus*, is placed in water in a watch-glass and then absolute alcohol is gradually added portion-wise and pouring away excess of the mixture at intervals until the fluid is finally all absolute alcohol. The preparation is then boiled in the alcohol and the greenish extract poured away, and this is repeated till the green tissue becomes colourless. The decolorised tissue is then brought back again into distilled water by gradually adding the water to the alcohol, and pouring off. Finally, it is allowed to stand a few minutes in a watch-glass in water redistilled from glass, and is then ready for staining.

In addition to the unmordanted simple aqueous solution of well-washed hæmatoxylin in $\frac{1}{2}$ -per-cent. concentration introduced by Macallum* the older histo-chemical tests for iron were also utilised, namely, potassium ferrocyanide and hydrochloric acid for ferric salts, potassium ferricyanide and hydrochloric acid for ferrous salts, and ammonium hydrogen sulphide in glycerine for both. In our opinion the Macallum test surpasses all these in reliability and delicacy. Its only fault is that it is too delicate, and the

* 'Journ. Physiol.,' vol. 22, p. 92 (1897).

small traces of inorganic iron set free from organic compounds in the tissues on long standing cause faint but increasing staining when a preparation is left over for some days. When a blue-black is obtained, however, within a few hours with this reagent it is a decisive proof of loosely combined, or inorganic, iron in the situation where the staining occurs.

Ammonium hydrogen sulphide when added to the tissues with an equal amount of glycerine, and the whole kept at 36° C. for some hours, produces a distinct blackening as compared with the normal, but the effect is not very pronounced and is only clear on comparison of treated and untreated tissue.

Potassium ferrocyanide and hydrochloric acid never gave a blue colour, but a blue colour is frequently, and very distinctly, given within a few hours by potassium ferricyanide and acid, demonstrating that the inorganic iron of the chloroplasts is present in the ferrous condition; this was typically observed in the case of *Spirogyra* and *Vaucheria*.

There is always some doubt, however, about using a reagent which itself contains the element sought for, and moreover is fairly readily broken down in presence of organic matter and acid.

The hydrochloric acid used should not exceed 0.5 per cent. in concentration, and be used in equal volume with the 1.5-per-cent. ferricyanide solution so that the concentration of acid acting on the tissue is only 0.25 per cent. Then, if a blue stain is obtained with a considerable intensity within 24 hours, it may fairly certainly be attributed to ferrous iron in that particular situation. The result, however, ought always to be confirmed by the Macallum test, for solid starch or casein left for 24 hours in contact with the above reagents each give a faint blue colour which increases as the mixture is left standing.

When a solution of hæmatoxylin in pure distilled water is mixed with a very dilute solution of an ordinary iron salt such as ferric chloride, a deep blue-black coloration is immediately produced. If, instead of an ordinary iron salt solution, a solution of highly colloidal or dialysed iron oxide be mixed with the solution of hæmatoxylin there is obtained instead a deep chocolate-brown coloration. In the course of some hours to a day or two, this chocolate brown is replaced by the blue-black colour obtained with ionic or crystalloidal iron salts. Similar results are usually obtained when the hæmatoxylin solution is used as a detector of iron in the tissue of plants. In certain cases, notably unicellular green plants and algal filaments, a deep blue-black is obtained within a few minutes without any previous appearance of the brown stain characteristic of colloidal iron oxide, while in many of the higher plants (mono- and dicotyledons) the green leaf at first stains

a deep brown, which gradually, in a varying period of a few hours to a day or two, changes to a blue-black, just as is seen in the test-tube when colloidal iron oxide solution is mixed with the reagent. In certain cases, however, the brown colour is found to persist for weeks without change.

This deep brown coloration is not simply due to imbibition of the tissue with unaltered hæmatoxylin, for it is far too deep for this, and, moreover, is not removed by washing with a mixture of equal parts of alcohol and ether as recommended by Macallum. It is a true staining of colloidal iron, present in those parts of the tissues where the brown occurs, and possesses just the same dark brown colour that is obtained on mixing colloidal iron oxide solution and hæmatoxylin.

In contrast with vegetable tissues, such a direct staining (either brown or blue-black) is only found in the embryonic condition in the tissues of higher animals, for the iron in the majority of such animal tissues is firmly bound organically and gives no coloration with hæmatoxylin.

It is to be remarked that this staining as a test for iron is quite different from the ordinary use of hæmatoxylin as a nuclear stain in histological technique. In the ordinary use of hæmatoxylin as a staining reagent a mordant is always used either preceding the hæmatoxylin, as, for example, the iron alum mordant for Heidenhain's iron-hæmatoxylin method, or simultaneously as in the use of the hæmalum stain, where the mordant alum is mixed with the hæmatoxylin. But in Macallum's use of the stain no mordant whatever is used, but instead a simple aqueous solution in pure distilled water. This solution only strikes a colour where a mordant is naturally present in the tissue. Now with iron in colloidal form the colour struck is the deep brown mentioned above, with iron in crystalloidal form the colour struck is blue-black. Thus Macallum's method resembles Heidenhain's staining, but with the previous iron treatment naturally provided in the tissues, and the blue-black effect obtained closely resembles in many cases a Heidenhain iron-hæmatoxylin stain.

In order to use the method effectively, it is not merely necessary to avoid all minute traces of iron in the water and other fluids used, but also all traces of alkali and acid, since these interfere with the delicacy of the reaction. Alkali gives a rose-red colour with the hæmatoxylin, and acid inhibits the development of the blue-black when the amount of iron is small. In making up the stain itself, water twice distilled from glass vessels must be used as the solvent, the second distillation having been made immediately previous to use. To make the staining solution, 0.3 grm. of pure hæmatoxylin is weighed out, and washed with the twice distilled water till the crystals are colourless, and the wash-water is only pale

yellow without any trace of red. The solution is then made up to 50 c.c. and kept in a Jena glass flask, for the alkali which is slowly dissolved out from ordinary glass rapidly turns the solution pink. The reagent should be pale yellow when used, in order to obtain the best effects, and does not keep in good condition for more than a few days.

After the chlorophyll and fats have been removed from the tissue by allowing it to stand in cold alcohol, or by boiling up with alcohol, the colourless tissue must be well washed with water, and the water used must, as described above, be doubly distilled from glass.

The staining process may be watched in progress, when it will be found that escaped chloroplasts from ruptured cells take on the stain first, and in many cases show a deep purple-blue within a few minutes. Within the intact cell the stain does not penetrate so rapidly, and the cell wall may show a blue staining in some cases before the contained chloroplasts, but eventually these also stain a deep blue, sometimes preceded by a dark brown. The nuclei of the green plant cells also stain a deep blue (unlike animal cell nuclei), and there is usually a much slighter diffuse blue in the remaining cytoplasm. The fibres associated with the vascular bundles also show in many cases a blue staining. This probably means that the iron salts are carried along this route to the green cells. But the early and deep staining of chloroplasts and nucleus are characteristic in the preparations.

In addition to tissues containing chloroplasts, several preparations have been made from plants not containing chlorophyll, such as yeast, moulds, and larger fungi. There is a marked contrast found here, a blue stain does not appear for some days, and then in only a comparatively feeble manner. The conidia and the conidiophores show more iron than the mycelium filaments. It is probable that this slow and feeble staining is due to organic iron compounds slowly breaking up and yielding traces of inorganic iron.

A series of ash analyses of chlorophyll-containing and chlorophyll-free plants show in all cases a much higher percentage of iron in the ash of the green plant; these analyses will furnish the subject of a separate paper.

A large number of plants of different types have been examined, and the main results are given in the following account.

Amongst unicellular green plants there were examined *Chlorella*, obtained as plankton from a green-coloured pond water; *Pleurococcus*, obtained in nearly pure condition growing on an oak fence near Oxford, and stained and examined in collaboration with Mr. Edward Whitley; several forms of diatom and several unicellular forms found in lichens.

The blue-black effect is very readily obtained with these unicellular green plants, often without previous removal of the chlorophyll. In the case of

the lichens the contrast is marked between the green cells and the fresh hyphæ of the fungus, but dead or decaying fungal matter often gives a blue stain.

The algae observed were species of *Vaucheria*, *Spirogyra*, *Ulva*, and *Ulothrix*. The effects were often repeated in several experiments, both with hæmatoxylin staining and with ferricyanide and hydrochloric acid. The ferricyanide solution does not appear to penetrate well, and only some filaments in an alga like *Spirogyra* are coloured, but the staining has been obtained within an hour or two of treatment with this reagent, and is a very beautiful effect when obtained in *Spirogyra*. The light blue colour follows the spirals of the chlorophyll bands, and the granules are obviously more deeply blue than the rest of the bands. The deep blue-black with hæmatoxylin is more readily and uniformly obtained, coming often within a few minutes of applying the stain to the decolorised alga, and furnishing again a beautiful effect. Sometimes, however, the brown colour of colloidal iron is obtained in *Spirogyra*.

Ulva latissima gives a very deep blue-black coloration, rendering the cells almost opaque; its ash shows a high content in iron.

Cladothrix, when growing in water containing small amounts of iron, as is well known, secretes, or excretes, a tube of iron oxide around the filaments, and is then known as an "iron bacterium." When these so-called "iron bacteria" are treated with hæmatoxylin, they turn blue-black almost instantly, and, if the stained specimens are examined under the microscope, the interesting fact is immediately observable that not only the external tube, but the substance of the organism itself, is stained blue-black, so settling a much disputed point. The same is seen in *Vaucheria*, an incrustation of iron-oxide particles is demonstrable in the gelatinous sheath surrounding the filaments, either by ferricyanide or hæmatoxylin staining, but, in addition, both reagents show inorganic iron within the filament itself.

Many higher aquatic plants, such as *Lemna* and *Elodea*, possess such incrustations of iron oxide on their leaves when grown in water containing only traces of iron, but in such cases it is also found that the chloroplasts of the green cell itself are very rich in inorganic iron. The higher aquatic plants examined have been these two and a variety of water-cress, and all three were found to give a strong positive reaction.

Ordinary lawn grass contains a high percentage of iron in the ash, and, when teased out and deprived of chlorophyll by hot alcohol, forms a very suitable object on account of the ease with which strands of fibre with attached cells separate. The staining of the chloroplasts is at first a dark brown passing later into a blue-black. The leaves of many species of

dicotyledonous plants were examined and it was found that here the transition from dark brown to blue-black was much slower as a rule, and in some cases the staining remained permanently of a deep orange-brown to a pure dark brown colour. But in all cases the chloroplasts stained more deeply than the remainder of the cytoplasm.

The catalyst for the photo-synthesis may not in all cases be an iron salt, or oxide, but an iron salt is present and capable of operating as a catalyst in a large number of instances.

Various substances known to be present in the ash of leaves have been tested for their photo-synthetic activity in connection with the work, and it has been found that magnesium and calcium phosphates and bicarbonates are entirely ineffectual, but that marked photo-synthesis of formaldehyde is obtained with chlorides or colloidal hydrates of iron or aluminium.

Summary.

1. Inorganic iron salts and iron or aluminium hydrates in colloidal solution possess the power of transforming the energy of the sunlight into chemical energy of organic compounds.

2. Inorganic iron, in crystalloidal or colloidal form, is present in the colourless part of the chloroplast of the green plant cell in many plants.

3. In the absence of iron the green colouring matter cannot develop in the leaf, although the green colouring matter itself contains no iron.

4. In the presence of sunshine, the iron-containing substance of the chloroplast develops the colouring matter, so that this itself is a product of photo-synthesis induced by the iron-containing compound.

5. These facts afford an explanation of chlorosis, and its cure by inorganic iron salts, and demonstrate that iron is a primary essential in photo-synthesis, and the production of chlorophyll.

6. The iron-containing substances of the colourless portion of the chloroplast, and the chlorophyll produced by them, then become associated in the functions of photo-synthesis as a complete mechanism for the energy transformation.

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